

PATENT  
Attorney Docket 4205.1US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL700256994US

Date of Deposit with USPS: September 21, 2000

Person making Deposit: Amanda Trulson

APPLICATION FOR LETTERS PATENT

for

DENDRITIC CELL ACTIVATED IN THE PRESENCE OF GLUCOCORTICOID  
HORMONES ARE CAPABLE OF SUPPRESSING ANTIGEN-SPECIFIC T CELL  
RESPONSES

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CELLS  
DENDRITIC ~~CELL~~ ACTIVATED IN THE PRESENCE OF  
GLUCOCORTICOID HORMONES ARE CAPABLE OF  
SUPPRESSING ANTIGEN-SPECIFIC T CELL RESPONSES

5           Reference to Related Application: This application claims  
priority from Provisional Application Serial No. 60/157,442,  
filed October 4, 1999.

10           Field: The invention relates to the field of medicine.  
More in particular the invention relates to the field of  
immunotherapy.

15           Background: The remarkable immunostimulatory properties of  
dendritic cells ("DC") reside in their ability to transport  
antigens from peripheral tissues to lymphoid organs where they  
present these antigens to T cells in an optimal costimulatory  
context. (1). To achieve this complex sequence of events, DC  
exist in different functional stages. Immature DC behave as  
sentinels in peripheral tissues where they efficiently capture  
intigens. Upon pathogen invasion, induction of protective T cell  
responses requires the activation of immature DC into mature  
20 immunostimulatory cells. DC activation is triggered in inflamed  
tissues by cytokines such as IL-1 and TNF-a and by bacterial  
components such as LPS (2, 3). Activated DC migrate to T cell  
areas in the lymph nodes while upregulating their costimulatory  
capacities and optimizing their antigen presenting functions.  
25 Upon interaction with antigen-specific T cells, DC activation is  
further completed through engagement of the receptor-ligand (1)  
pair CD40-CD40L, leading to the production of IL-12 (4, 5, 6),  
a key cytokine for T helper (Th) type 1 and cytotoxic T  
lymphocyte (CTL) priming (7).

30           APC activation through CD40-CD40L interactions represents  
an important immunoregulatory step for the establishment of  
protective T cell immunity against pathogens and tumors (8, 9,  
10). This process also plays a key role in the onset of  
destructive T cell-mediated disorders such as auto-immune  
35 diseases, allograft rejection and graft versus host disease (11,  
12, 13). The current treatment of these

disorders largely relies on the administration of  
 glucocorticoids (GC), which exert potent anti-inflammatory  
 and immunosuppressive effects. Because GC negatively  
 interfere with many aspects of T cell activation such as IL-  
 2-driven proliferation and inflammatory cytokine production  
 (reviewed in 14), activated T cells have long been considered  
 as the main targets for GC action. Several lines of evidence  
 now suggest a role for DC in GC-induced immune suppression.  
 Moser et al (15) found that GC prevented the spontaneous  
 activation of murine DC thereby decreasing their T cell  
 stimulatory potential. Kitajima et al (16) showed that GC  
 could hamper the T cell-mediated activation of a murine DC  
 line. Viera et al reported that human DC exposed to GC were  
 poor producers of IL-12 upon LPS stimulation (17). These  
 findings only concern loss of typical DC features and  
 therefore favor a simple inhibitory role of GC on DC  
 activation. A more complex immunoregulatory action on the DC  
 system has not been considered.

The present invention resulted from a detailed analysis of  
 the impact of GC on the CD40-mediated activation of monocyte-  
 derived DC. These DC develop after culture with GM-CSF and  
 IL-4 (2, 18) or after transmigration through endothelial  
 cells (19) and are known to mature into the most potent human  
 Th1-type-inducing APC upon CD40 ligation (5, 20). Moreover,  
 these APC can easily be generated in large numbers and are  
 thereby the cells of choice for DC-based modulation of T cell  
 immunity (21, 22). In contrast to previous studies, the  
 present invention shows that GC such as dexamethasone (DEX)  
 do not merely prohibit DC activation, but that it converts  
 CD40 ligation on human monocyte-derived DC is transformed  
 into an alternative activation pathway. DEX profoundly affect  
 the CD40-dependent maturation of human monocyte-derived DC,  
 not only by preventing the upregulation of costimulatory,  
 adhesion and MHC surface molecules, but also by causing these  
 cells to secrete the anti-inflammatory mediator IL-10 instead  
 of the Th1 stimulatory cytokine IL-12. In agreement with

these phenotypic and functional changes, DC triggered through CD40 in the presence of DEX are poor stimulators of Th1-type responses. Most importantly, the present invention shows that such DC are able to induce a state of hyporesponsiveness in Th1 cells, indicating that these cells are capable of active suppression of Th1-type immunity.

#### *Summary of The Invention*

As already mentioned above, the impact of GC on DC has been the subject of several previous studies by others. However, in contrast with the present invention, these studies only highlighted inhibitory effects of GC on the DC system. DEX was found to block the upregulation of CD80, CD86 and MHC class II molecules upon activation of murine spleen DC (15, 16), whereas very recently DEX was demonstrated to also prevent the differentiation of DC from monocyte precursors (28). In these studies, the inability of DC to acquire high expression of costimulatory and MHC molecules was accompanied with a decrease in their T cell stimulatory potential, but the effect of GC on IL-12 production was not investigated. On the other hand, Viera et al found that the effect of GC on LPS-induced DC activation consisted in a 4-fold reduction of IL-12p70 synthesis (17). This partial effect on IL-12 secretion contrasts with the complete suppression of IL-12p70 production which is subject of the present invention, and can be explained by the fact that their GC-treated immature DC were extensively washed prior to LPS stimulation. We indeed found that upon removal of GC, the effects of these drugs on immature DC were rapidly reversible. The continuous presence of GC during CD40 triggering of DC was clearly preferred in order to stably and completely modulate DC activation (data not shown). Taken together, previous findings indicated that the impact of GC on the DC system should be merely interpreted as an inhibitory event. Importantly, the present invention clearly demonstrates that GC such as DEX do not simply suppress DC activation but rather redirect this process towards a distinct functional program.

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said activated dendritic cells with an antigen against which said T cell response is to be reduced. An unwanted T cell response can be any type of T cell response. For instance, but not limited to, a T cell response associated with an auto-immune disease or a transplantation disease such as a graft versus host disease or a host versus graft disease. A pharmaceutical composition of the invention typically comprises a dendritic cell of the invention suspended in a liquid suitable for preserving the function of said dendritic cell in said liquid and/or suitable for administration to a host. A host preferably is a human. Preferably said host is at risk of developing or is suffering from an auto-immune disease or allergy. Preferably, said host suffers from or is at risk of suffering from host versus graft disease and/or graft versus host disease. With the term <sup>at risk, it</sup> ~~at risk~~ is meant that one expects that said host may develop said disease, for instance but not limited to a host receiving a transplant. Such a host is considered to <sup>be</sup> ~~at~~ risk of developing host versus graft disease. An antigen typically is a peptide capable of binding to a major histocompatibility complex I and/or II molecule. Such peptides are known in the art and a person skilled in the art is capable of determining whether a given peptide comprises an antigen or not. An antigen may be derived from a naturally occurring protein. An antigen may also be a synthetic peptide or equivalent thereof, preferably with an amino-acid sequence equivalent to a peptide derived from a protein.

In another aspect, the invention provides a pharmaceutical composition for reducing an unwanted T cell response in a host, said composition being obtained by culturing peripheral blood monocytes from said host to differentiate into dendritic cells, activating said dendritic cells in the presence of a glucocorticoid hormone and loading said activated dendritic cells with an antigen against which said T cell response is to be reduced. In one embodiment, a method is provided for reducing an unwanted T cell response in a

The invention further provides method for reducing an unwanted T cell response in a host comprising culturing

10 administering said composition to said host.

20 In another aspect, the invention provides a method for  
obtaining an dendritic cell capable of tolerising a T-cell  
for an antigen comprising providing said dendritic cell with  
a glucocorticoid hormone, activating said dendritic cell and  
providing said dendritic cell with said antigen. With the  
25 term tolerising is meant that said dendritic cell has an  
immunosuppressive effect on said T cell. A tolerised T cell  
will essentially not respond with cell division when exposed  
to a cell presenting an antigen said T cell would in the  
untolerised state respond to with cell division. A tolerised  
0 T cell will essentially not respond with killing a cell  
presenting an antigen said T cell would in the untolerised  
state respond to with cell kill.

In one embodiment, said dendritic cell and/or a precursor thereof is provided with said glucocorticoid hormone in vitro. A T cell of the invention is preferably an antigen specific T cell, preferably a cytotoxic T cell or a Th cell.

Method of treatments of the invention are preferably <sup>used</sup> ~~use~~ for the treatment of an individual suffering from an auto-immune disease, an allergy, a graft versus host disease and/or a host versus graft disease.



## Examples

### Example 1

#### *Impairment of CD40-CD40L-mediated phenotypic changes by DEX*

5 We explored the impact of DEX on the phenotypic changes induced by CD40 ligation on immature monocyte-derived DC. In the absence of DEX, the fusion protein CD8-CD40L induced a strong upregulation of the costimulatory molecules CD80, CD86 and CD40, of the MHC class I and II molecules, of the

10 adhesion markers CD54 and CD58 and of the DC maturation marker CD83 (Fig 1). In the presence of DEX, these CD8-CD40L-induced phenotypic changes were dramatically impaired: the upregulation of CD80, CD86, CD40, CD54, CD58 and of the MHC class I and II molecules was largely inhibited and CD83 was

15 not expressed (Fig 1). Importantly, DEX-treated DC did not revert to a monocyte/macrophage stage as shown by the lack of expression of CD14 (Fig 1). Titration of DEX showed a complete inhibition of CD40-mediated phenotypic changes at  $10^{-6}$  M and  $10^{-7}$  M, a partial blockade at  $10^{-8}$  M and no effect

20 at  $10^{-9}$  M and  $10^{-10}$  (data not shown). In addition, DEX action was dependent on binding to the GC-receptor, since it was abolished by simultaneous addition of the GC receptor antagonist RU486 (data not shown). In experiments performed with LPS or TNF- $\alpha$  as activation agents, similar results were

25 obtained. However, the combination of DEX and TNF- $\alpha$  induced a massive cell death (viable cell recovery 5-10% of control cultures), a phenomenon that was not observed when DEX-treated DC were stimulated with LPS or through CD40 (viable cell recovery 60 to 100% of control cultures) (not

30 shown).

We next analyzed whether activated DC could still be affected by DEX. DC incubated with CD8-CD40L for 48h and further exposed to DEX maintained a stable activated phenotype (Fig 2).

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We conclude that DEX prevents the phenotypic changes induced by CD40 signals on immature DC and that already activated DC are resistant to DEX action.

#### 5 Example 2

*DEX does not interfere with the regulation of DC antigen uptake machinery*

Unlike activated DC, immature DC efficiently internalize antigens through macropinocytosis and mannose receptor-mediated endocytosis (2, 3, 25, 26). We analyzed whether DEX could affect the DC antigen capture machinery and its downregulation following CD40 cross-linking. As shown in Fig 3, incorporation of FITC-BSA and FITC-mannosylated BSA by immature DC and by DEX-treated immature DC was comparable. Upon CD40 triggering, a similar decrease of FITC-BSA and FITC-mannosylated BSA uptake by both DEX-treated and untreated DC was observed (Fig 3). These results were the first to indicate to us that DEX does not block all aspects of DC activation, since it does not interfere with the down-regulation of the DC antigen capture machinery.

#### Example 3

*DEX-treated CD40-triggered DC secrete IL-10 instead of IL-12*

A key feature of CD40-triggered DC for initiating T cell immunity resides in their ability to produce the proinflammatory cytokine IL-12 (5, 6, 27). We investigated whether DEX affected IL-12 production by DC stimulated through CD40, and we explored the possibility that DEX could promote the secretion of the anti-inflammatory cytokine IL-10. As shown in Fig 4, CD40 triggering of DC strongly induced IL-12p40 and IL-12p70 secretion (up to 120ng/ml and 170pg/ml respectively) but only poorly stimulated the production of IL-10 (up to 68pg/ml). In contrast, CD40 triggering of DEX-treated DC resulted in a dramatically reduced IL-12p40 production (up to 100 fold) and in the complete suppression of IL-12p70 secretion, whereas IL-10 production was strongly

enhanced (up to 50 fold) (Fig 4). Immature DC and their DEX-treated counterparts failed to secrete detectable amounts of IL-12 and IL-10 (Fig 4). Therefore, CD40 ligation of DC in the presence of DEX triggers the secretion of high levels of the anti-inflammatory cytokine IL-10 instead of IL-12.

#### Example 4

*DEX-treated CD40-triggered DC are capable of suppressing Th1-type immunity*

The strikingly modified response of DC to CD40 ligation in the presence of DEX prompted us to compare the T cell stimulatory potential of these cells with that of their DEX-untreated counterparts. In an allogeneic MLR, CD40-triggered DC induced a strong proliferative T cell response whereas the addition of DEX prior to CD40 triggering reduced their T cell stimulatory capacity to that of immature DC (Fig 5). When tested for their ability to stimulate an hsp65-specific CD4<sup>+</sup> Th1 clone, CD40-triggered DC pulsed with the hsp65 protein or with the specific peptide epitope p3-13 were found to be potent inducers of both T cell proliferation and T-cell dependent IFN-g production (Fig 5). In contrast, in the presence of Ag-pulsed DEX-treated CD40-triggered DC, T cell proliferation and IFN-g production were significantly decreased ( $p < 0.001$  and  $p < 0.01$  respectively) (Fig 5). We next investigated whether DEX-treated CD40-triggered DC were simply poor stimulators of Th1 cells, or whether they could exert suppressive effects on these T cells. We therefore tested hsp65-specific T cells stimulated with p3-13-pulsed DEX-treated CD40-triggered DC for their capacity to respond to a second potent antigenic challenge. Fig 6 shows that preculturing T cells with CD40-triggered DC led to a strong T cell proliferation and IFN-gamma production upon second antigen-specific restimulation. In contrast, preculture with DEX-treated CD40-triggered DC resulted in a dramatically reduced proliferative and IFN-gamma production capacity of Th1 cells. Thus, CD40 triggering of DC in the presence of DEX

results in APC that are not merely poor inducers of T cell responses but that also induce a state of hyporesponsiveness in Th1 cells.

## 5 Materials and Methods

### *Generation of DC*

Immature DC were generated from peripheral blood monocyte precursors. Human PBMC from healthy donors, isolated through

10 Ficoll-Hypaque density centrifugation were plated at  $1.5 \times 10^7$  per well in 6-well plates (Costar Corp., Cambridge, MA) in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 2mM glutamine, 100UI/ml penicillin and 10% FCS. After 2

h at  $37^{\circ}\text{C}$ , the non adherent cells were removed and the  
15 adherent cells were cultured in medium containing 500U/ml IL-4 (Pepro Tech Inc. Rocky Hill, NJ) and 800U/ml GM-CSF (kindly provided by Dr S. Osanto, LUMC, Leiden, NL) for a total of 7 days.

### 20 *Activation of immature DC with a CD8-CD40L fusion protein*

Activation of DC through CD40 was performed with a fusion protein made of the extracellular domain of human CD40L and of the murine CD8a chain (CD8-CD40L). The CD8-CD40L cDNA described by Garrone et al (23) was transferred into an  
25 eukaryotic expression vector containing the hygromycin resistance gene, and used for the generation of stably transfected Chinese Hamster Ovary (CHO) cells. Culture supernatants containing the CD8-CD40L fusion protein were concentrated with a pressurized stirred cell system (Amicon, Inc., Beverly, MA), checked for binding to CD40 and tested  
30 for optimal DC activation conditions (not shown). DC were incubated at  $5 \times 10^5$ /ml/well in a 24-well plate (Costar Corp., Cambridge, MA) and activated in the presence of 1/10 CD8-CD40L supernatant. Cells and supernatants were analyzed  
35 after 48 h. Of note, control supernatants obtained from

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untransfected CHO cells or from CHO cells transfected with the CD8a cDNA lacked DC activating functions and were similar to culture medium.

##### 5 *DEX and RU486 treatment of DC*

Seven days immature DC were treated with  $10^{-6}$  M DEX (Sigma, St Louis, MO) in the presence of GM-CSF and IL-4 or GM-CSF alone. After 24 h, DC were analyzed or were further stimulated via CD40 by adding the CD8-CD40L fusion protein to the cultures as described above. In some experiments, the glucocorticoid receptor antagonist RU485 (Roussel-UCLAF, Romainville, France) was used at 10mM final concentration, alone or in combination with DEX.

##### 15 *Analysis of DC surface phenotype by flow cytometry*

Cells were stained on ice with FITC or PE-conjugated mouse monoclonal antibodies (MoAb) for 30 min in PBS 1% FCS and were analyzed on a FACScan<sup>®</sup> (Becton Dickinson, San Jose, CA). The following MoAb were used: FITC-anti-CD80 (BB1), PE-anti-CD86 (FUN-1), FITC-anti-CD40 (5C3), PE-anti-CD54 (HA 58) and PE-anti-CD58 (1C3) (Pharmingen, San Diego, CA), PE-anti-CD14 (L243) and PE-anti-HLA-DR (Mf-P9) (Becton Dickinson), PE-anti-CD83 (HB15A) (Immunotech, Marseille, France) and PE-anti-HLA class I (Tu 149) (Caltag Laboratories, Burlingame, CA).

##### *Antigen uptake experiments*

DC were resuspended in medium buffered with 25mM Hepes. FITC-BSA and FITC-mannosylated BSA (both from Sigma) were added at 1mg/ml final concentration and the cells were incubated at 37<sup>0</sup>C, or at 0<sup>0</sup>C to determine background uptake. After 1 h, DC were washed extensively with iced-cold PBS and analyzed by FACS<sup>®</sup> using propidium iodide to eliminate dead cells.

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### *Cytokine detection by ELISA*

Culture supernatants were analyzed in serial twofold dilutions in duplicate. IL-12p70 was detected using a solid phase sandwich ELISA kit (Diacclone Research, Besancon, France) (sensitivity 3pg/ml). For IL-12p40 and IFN-g detection, capture MoAb and polyclonal biotinylated detection Ab were obtained from Peter van de Meijde (BPRC, Rijswijk, NL) (sensitivity 10pg/ml). IL-10 was detected using the Pelikine compact human IL-10 ELISA kit (CLB, Amsterdam, NL) (sensitivity 3pg/ml).

### *Allogeneic mixed lymphocyte reaction (MLR)*

Non adherent allogeneic adult PBMC from an unrelated individual were cultured in 96-well flat-bottom plates (Costar Corp., Cambridge, MA) at a density of  $1.5 \times 10^5$ /well with various numbers of g-irradiated (3,000 rads) DC, in triplicates. Proliferation was assessed on day 5 by [ $^3$ H]thymidine uptake (0.5mCi/well, specific activity 5Ci/mMol, Amersham Life Science, Buckinghamshire, UK) during a 16 h pulse.

### *Th1 stimulation assays*

The Mycobacterium tuberculosis and M. leprae hsp65-specific, HLA-DR3-restricted CD4+ Th1 clone Rp15 1-1 used in this study recognizes an hsp65 determinant corresponding to peptide residues 3 to 13 (p3-13) (24). HLA-DR-matched DEX-treated immature DC and their DEX-untreated counterparts were pulsed with 10mg/ml of p3-13 or with 10mg/ml of hsp65 for 2 h, washed extensively and stimulated through CD40 as described above. For Ag-pulsed DEX-treated immature DC, CD40 triggering was performed in the presence of DEX. Hsp65 specific T cells ( $10^4$ ) were cultured with different numbers of g-irradiated (3,000 rads) DC in 96-well flat-bottom plates (Costar Corp.) in triplicates for 3 days. [ $^3$ H]thymidine incorporation was measured on day 3 after a 16 h pulse. Before the addition of

[<sup>3</sup>H]thymidine, 50ml of supernatants were collected from each well and supernatants from triplicate wells were pooled to measure IFN-g production. To test hsp65-specific T cells responsiveness to a second potent antigenic challenge, 10<sup>4</sup> T cells were first cultured for 48h with 5 x 10<sup>3</sup> peptide-pulsed DC prepared as above, then harvested and allowed to rest in medium containing 5U/ml IL-2. Three days later, 10<sup>4</sup> viable T cells were restimulated with 5 x 10<sup>3</sup> peptide-pulsed DC generated from the same donor as used for the first culture and tested for their ability to proliferate and to produce IFN-g as previously described.

#### Statistical analysis

Covariance analysis was used to compare T cell proliferation and IFN-g production as a function of DC number, between DEX-treated CD40-triggered DC and DEX-untreated CD40-triggered DC (Fig. 5).

Fig. 5a: 10<sup>4</sup> T cells

Fig. 5b: 10<sup>5</sup> T cells

Fig. 5c: 10<sup>6</sup> T cells

Fig. 5d: 10<sup>7</sup> T cells

Fig. 5e: 10<sup>8</sup> T cells

Fig. 5f: 10<sup>9</sup> T cells

Fig. 5g: 10<sup>10</sup> T cells

Fig. 5h: 10<sup>11</sup> T cells

Figure legends

Fig. 1 Pretreatment with DEX inhibits the phenotypic changes induced by CD40 ligation.

- 5 Seven days immature DC were cultured for 24h in the absence or the presence of  $10^{-6}$ M DEX and activated via CD40 with the CD8-CD40L fusion protein for 48h. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls
- 10 MoAb and solid histograms represent specific staining of the indicated cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 4. Data are representative of 4 independent experiments.

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Fig. 2 DC triggered through CD40 maintain an activated phenotype upon a subsequent DEX exposure.

- Immature DC were activated with the CD8-CD40L fusion protein.
- 20 DEX ( $10^{-6}$ M) or medium control were added 48h later and cells were analyzed after 2 additional days of culture. The comparison with immature DC maintained in medium alone is shown. Empty histograms show the background staining with isotype controls MoAb and solid histograms represent specific
- 25 staining of the indicated cell surface markers. Specific mean fluorescence intensities are indicated. Mean fluorescence intensities of isotype controls were between 3 and 5. Data are representative of 2 independent experiments.

- 30 Fig. 3 Pretreatment with DEX does not affect the regulation of DC antigen uptake machinery.

Immature DC were incubated in the absence or the presence of  $10^{-6}$ M DEX for 24h and further activated or not via CD40 with the CD8-CD40L fusion protein for 48h. Cells were pulsed for

35 1h with medium containing either 1mg/ml FITC-BSA or 1mg/ml



FITC-mannosylated BSA. Empty histograms show the background autofluorescence, Grey-filled histograms show the background uptake at 0°C and black-filled histograms show the specific uptake at 37°C. Data are representative of 3 independent experiments.

**Fig. 4** Pretreatment with DEX alters the cytokine secretion profile of CD40-triggered DC.

DEX-exposed or control immature DC were left in culture without further treatment or stimulated with the CD8-CD40L fusion protein. Culture supernatants were harvested 48h later and IL-10, IL-12p40 and IL-12p70 secretion were analyzed by specific ELISA. Data are representative from 6 independent experiments.

**Fig. 5** Pretreatment with DEX impairs the T cell stimulatory capacities of DC activated via CD40 and leads to a state of hyporesponsiveness of Th1 cells.

Allogeneic MLR: non adherent allogeneic PBMC were cultured with different numbers of CD40-triggered DC, DEX-treated CD40-triggered DC or immature DC. The proliferative response was measured on day 5.

Th1 stimulation assays: Hsp65-specific T cells were cultured with different numbers of HLA-DR matched CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the hsp65 protein or with the specific p3-13 peptide epitope. The proliferative response and the T cell dependent IFN- $\gamma$  production were analyzed on day 3. Data are representative of 4 independent experiments.

**Fig. 6** DEX-treated DC triggered through CD40 induce a state of hyporesponsiveness in Th1 cells. Hsp65-specific T cells precultured with CD40-triggered DC or with DEX-treated CD40-triggered DC pulsed with the p3-13 peptide epitope were harvested after 48h, allowed to rest in the presence of 50U/ml

~~IL2 for 3 days, and restimulated with p3-13-pulsed DC. The~~  
~~proliferative response and IFN- $\gamma$  production were measured on~~  
~~day 3. Similar results were obtained in 2 independent~~  
~~experiments.~~

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p3-13-pulsed  
 DC stimulation  
 in 2 independent

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